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Une micro-methode de l'immuno-electrophorese

A Micro-Method of Immuno-Electrophoresis

by
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A scant two years ago a preliminary note of Grabar and Williams [2] appeared, dealing with immuno-electrophoretic analysis in agar medium. Since that time the method has been developed both at the Pasteur Institute, Paris, and the Polyclinic of Medicine, Geneva. C.A.Williams made it the subject of a thesis, and the results obtained have been published in various articles.[1,3-8,10,13] We shall return to what has already been published only to illustrate the micro-method presented here, and we refer to the articles cited for all details concerning the original method.

Let us recall here the principle of it. A mixture of antigens is separated electrophoretically in agar gel, after which a corresponding immune serum is allowed to diffuse in the gel itself.

The occurrence of an antigen with its homologous antibody provokes the formation of a linear precipitate, as there are different antigens in the mixture analyzed. The method permits, on the one hand, confirming the individuality and the homogeneity (or heterogeneity) of electrophoretic fractions, and, on the other, attributing a definite electrophoretic mobility to the different known antigens.

We quickly found that the originalmethod was too difficult and did not permit undertaking research on rare material. In addition, after a certain time, we found ourselves short of immune serum.

Consequently, it was the impossibility of obtaining a sufficient quantity of certain antigens and the lack of antiserum that determined our working out this micro-method which answers the evident need.

In the table below we sum up the differences presented between the standard method and the micro-method for an analysis of human serum made with the help of the same immune serum of horse.

	Volume in ml. of		Time	
	Serum	Immune serum	Electrophoresis	Diffusion
Standard Method	0.05-0.2	0.05- 1.0	6 hrs. for 3 V/cm.	1-10 days
Micro-method	0.001	0.005	45 min. 6 V/cm	½-24 hrs.

It can be seen that the reduction is of the order of a one hundredth.

Equipment

A. Tank for electropherisis of the paper electrophoresis type with horizontal bands, or better: a special tank with distance between the receptacles at the electrodes reduced.

B. Continuous voltage generator, producing sufficient voltage, (approximately 200 V).

C. Microscope slides of the current type (76/26mm.).

D. Punch to make holes in the agar. A very simple method consists of inserting into a large cork stopper, about 7 mm. from one another, two no. 10 hypodermic needles, the feather edge of which has been ground down. Two razor blades are forced into the cork stopper, separated by a wedge of suitable thickness (about 1 mm). It is well to shorten the blades in a manner to obtain a cut 3 cm. in length. This instrument, pressed on the agar, cuts two openings, which will receive the antigens, and a median slit destined to receive immune serum. This procedure assures the constant arrangement indispensable to good reproducibility. We improved the apparatus by holding the assemblage above at the end of a level joined to a small board. The two needles are connected to the water suction pump and aspiration is effected immediately.

Technique*

The gel used is 2% of $_{p}H$ 8.2, and ionic strength of .05. A 4% agar gel is prepared in distilled water: then for use, it is diluted while warm with an equal volume of veronal buffer of $_{p}H$ 8.2 and ionic strength of .1. After filtration, while warm, exactly 2 ml. of this agar is cooled for each slide. The agar spreads regularly on the slide and is kept there while forming a meniscus on the edges. By cooling a gel is obtained about 1 mm. in thickness. With the help of the punch the holes are marked then, through a fine pipet, the small plug of agar cut out by the two needles is aspirated by the water suction pump. The median band still remains in place, the two holes are filled with the antigen solutions to be inquired into. The slides so prepared are placed flat in the electrophoresis apparatus. At each end of the slides a band of filter paper assures contact between agar and the electrode vessels. A voltage of 40 volts is applied to the end of the slides, which corresponds to about 6 V/cm. Naturally we can work with a lower voltage by proportionately extending the time of electrophoresis. The voltage to be applied to the generator terminals depends on the dimensions of the electrode vessels (labyrinths, etc.) and the length of the paper bridges. To avoid too great a drop of voltage in the electrodes, we fill them with more concentrated buffer (u 0.1). As soon as the serum has penetrated into the agar, the hole is stopped up again by pouring warm agar into it.

* Note: Our thanks go to Miss H. Toulet, Laboratory assistant, for her valuable collaboration.

In 45 minutes the separation is completed. The slides are withdrawn, the median band of cut out agar is aspirated, and the cut so formed is filled with .005 ml. of immune serum, on occasion diluted to suitable proportions. The diffusion is very rapid, and at the end of half an hour the first lines are seen to appear. After 12 hours the image can be considered as complete.

Recording of Images

The preparations can be photographed either by direct contact on paper or film or by using the slide as negative in a magnifying apparatus, or finally by film exposure by means of an objective, (an apparatus for small form and size, 24 x 36 mm., lateral illumination on a black background).

It seemed handy to us to proceed in the following manner: after 7-8 hours of diffusion a film photograph is taken which permits locating certain antigens, the precipitates of which, soluble in an excess of antigen, will disappear later on, for example albumin. After 24 hours of diffusion the precipitates are fixed as follows: a thick blotter, applied to the agar, absorbs the water with the salts and proteins in solution. The precipitated proteins remain in the agar. After complete drying a film of agar is left adhering tightly to the glass[11]. The precipitates, became transparent, are marked in relief. The plates as prepared lend themselves well to preservation. Their

opacity can be restored to the lines at any time by moistening them with water. Nevertheless, it is preferable to make them visible in a positive way by staining them with an appropriate dye. Proteins, for instance, can be stained by plunging the preparation, dried and remoistened as described above, for one minute in a solution saturated with amido black 10 B. (100mg. of dye per 100 ml. of mixed methanol-acetic acid 9-1). Rapid washing in this solvent and drying in air terminate the operation. Staining with azocarmine (saturated solution in methanol containing 3% acetic acid) likewise produces beautiful preparations easier to photograph.

Immuno-Electrophoretic Analysis of Normal Serum

Results and Commentaries:

We have described elsewhere[8] the image that normal human serum gives by the original method. Fig. 1 represents schematically the lines produced by normal antigens of human serum with respect to an immune serum of horse anti-human serum.

The order of appearance of the lines as a function of the time is determined by the respective concentrations and the diffusion rates of the antigens.(fig. 2)

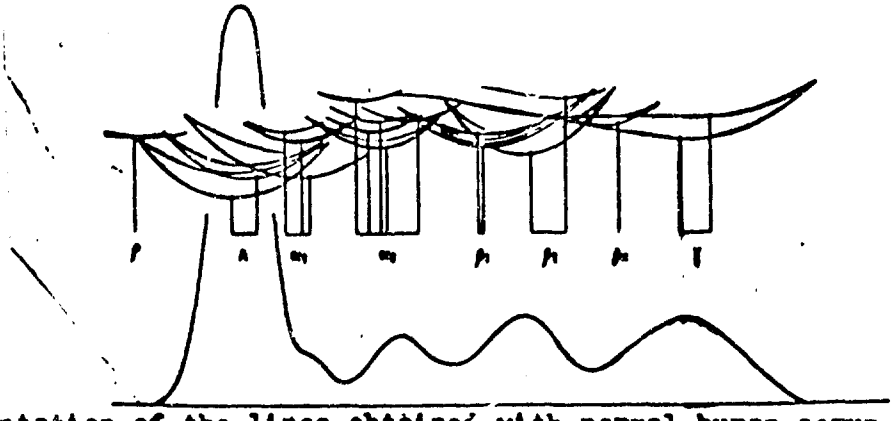
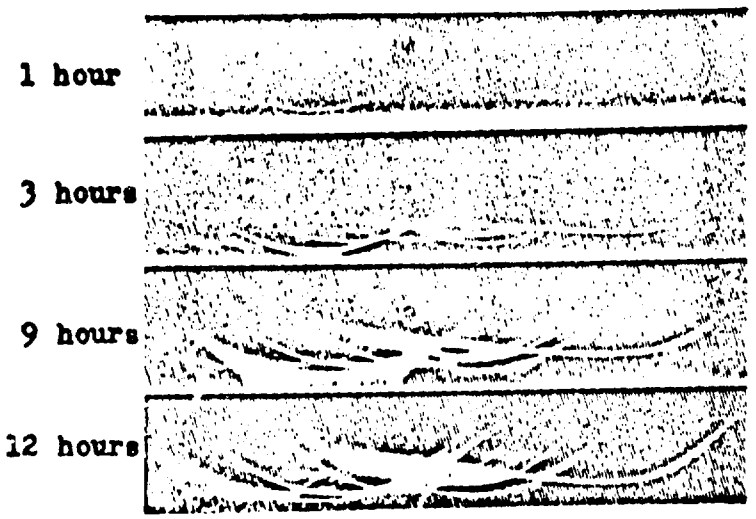


Fig.1. Schematic representation of the lines obtained with normal human serum by an immune serum of horse anti serum(above) and correspondance with classical electrophoresis (below). Date June 1954.

First albumin is seen to appear, then the other fractions at the same time that the albumin spreads and obliterates.

Fig. 2 Development of precipitates as a function of time. In 24 hours there is a complete image.



The diffusion time is extremely short in regard to the original method. In many cases we have the result by evening when getting the experiment underway in the morning. This is only in case of a complex mixture, such as serum, for which it is necessary to wait 12 hours or more!

The effect of the concentration in antigens is illustrated by Fig.3, showing a series of dilutions. The volumes used, as well as the antigen-antibody ratios, are the following:

Photo.	a	b	c	d	e	f	g	h	i
Vol. antigènes mm³ . . .	1	1	1	1	1	1/2*	1/4*	1/6*	1/6*
Vol. immunsérum mm³ . .	1	2	3	4	5	5	5	5	15
Rapport	1/1	1/2	1/3	1/4	1/5	1/10	1/20	1/30	1/90

*Actually we took 1 mm^3 of serum diluted in the proportion 1/2, 1/4, 1/6.

Scheidegger, Une micro-méthode de l'immuno-électrophorèse

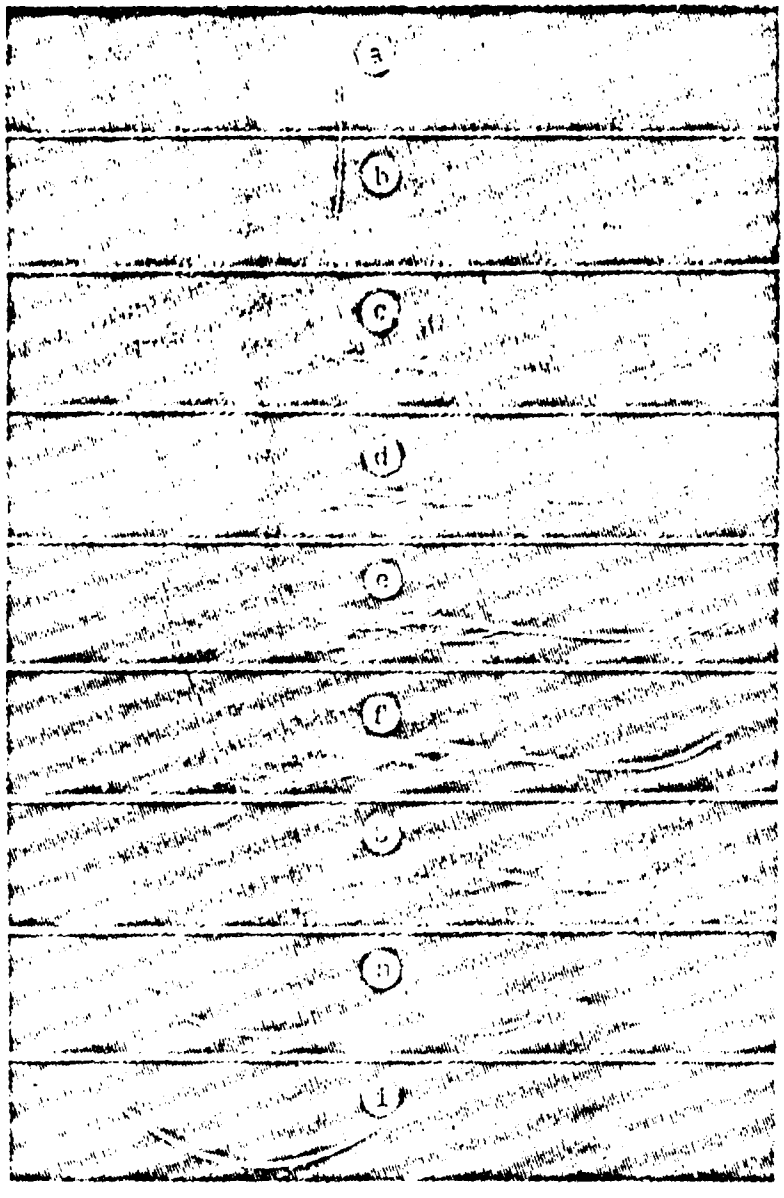


Fig.3. Variation of relative concentrations of components. From top to bottom: excess of antigens, optimum, excess antiserum. Explanation in text and above table.

In the particular case of the immune serum of horse anti-human serum which we use, (the serum of the Pasteur Institute, Paris, was kindly put at our disposa; by Prof. Grabar, whom we warmly thank), the optimum ratio is 1 mm^3 of human serum to 5 mm^3 of immune serum (photo "e"). It is there we observe the greatest number of lines. Above fractions are found for the most part an excess of antigens. In "d" the albumin is already no longer visible, and the more we go up the fewer lines remain. Below "e" there is an excess of antibldy for a great number of fractions.

It is from "f" and "g" we see best the rho fraction, wheras the albumin approaches equivalence only in the last dilution, where all other lines have disappeared or almost disappeared.

Indeed, the response of the animal to immunizing injections is not proportional to the amounts of antigen received. Thus the amount of anti-albumin is far lower than that of the anti-gamma, although in the immunizing serum the albumin represents about triple the gamma globulins.

In view of the very small volume of serum used, nothing opposes putting on both sides of the immune serum different amounts of one and the same mixture of antigens. Thus, we obtain for routine analysis two images which supplement one another.

Fig. 4. Routine image. Above we placed 1 mm^3 of normal human serum: below, the same volume of serum diluted to one fourth. Between them we put 5 mm^3 of immune serum. The length of the cut is 3 cm. The slight distortion which spreads toward the middle portion is sometimes produced at drying and has no apparent effect on the form of the lines.

A new possibility is likewise opened by this method. It is no longer necessary to take blood by venipuncture. We can take blood by pricking the end of a finger and collecting it in a capillary tube, where we can let it coagulate. After the clot is retracted, we break the point of the capillary and let the serum run out into the hole. Some red corpuscles that are possibly carried along do not hinder. This advantage permits using immune-electrophoretic analysis first in pediatrics, then likewise at experimentation in all small animals.

Summary

The micro-method of immuno-electrophoretic analysis shows the following advantages over the original method:

1. Economy of immune serum.
2. Economy of time.
3. It is less cumbersome.
4. A micro-drop of serum may be examined allowing it to be used in pediatrics and animal experimentation.

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